Antibiotics as intermicrobial signaling agents instead of weapons

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It has been widely assumed that the ecological function of antibiotics in nature is fighting against competitors. This made them a good example of the Darwinian struggle-for-life in the microbial world. Based on this idea, it also has been believed that antibiotics, even at subinhibitory concentrations, reduce virulence of bacterial pathogens. Herein, using a combination of genomic and functional assays, we demonstrate that specific antibiotics (namely tobramycin, tetracycline, and norfloxacin) at subinhibitory concentrations trigger expression of determinants influencing the virulence of the major opportunistic bacterial pathogen *Pseudomonas aeruginosa*. All three antibiotics induce biofilm formation; tobramycin increases bacterial motility, and tetracycline triggers expression of *P. aeruginosa* type III secretion system and consequently bacterial cytotoxicity. Besides their relevance in the infection process, those determinants are relevant for the ecological behavior of this bacterial species in natural, nonclinical environments, either by favoring colonization of surfaces (biofilm, motility) or for fighting against eukaryotic predators (cytotoxicity). Our results support the notion that antibiotics are not only bacterial weapons for fighting competitors but also signaling molecules that may regulate the homeostasis of microbial communities. At low concentrations, they can even be beneficial for the behavior of susceptible bacteria in natural environments. This is a complete change on our vision on the ecological function of antibiotics with clear implications both for the treatment of infectious diseases and for the understanding of the microbial relationships in the biosphere.

environment | hormesis | signals | antibiotics | *Pseudomonas aeruginosa*

It has been earlier stated that the amount of bacteria causing infectious diseases in the soil is low. This led to the idea that the soil could be a source of inhibitors of bacterial pathogens (1). Based in this idea, soils have been searched to find those inhibitors, and antibiotics have been discovered and further developed as bacterial killers. This finding led to the generalized assumption that such searched-on-purpose property was their actual function in nature: a part of the microbial armamentarium to fight competitors. Based on this belief, it has been frequently thought that antibiotics, even at subinhibitory concentrations, should necessarily reduce bacterial fitness and, in the case of bacterial pathogens, virulence. Although a number of previous publications seemed to support this classic view, the results usually are fragmentary and sometimes contradictory. In fact, a recently published work has shown that specific subinhibitory concentrations of the aminoglycoside tobramycin enhances the formation of bacterial biofilms (2), evidence suggesting that antibiotic-induced reduction of bacterial fitness (and virulence) is not always the result upon antibiotic exposure. To address in more detail the effect of subinhibitory concentrations of antibiotics in bacterial physiology and virulence, we have used as a model *Pseudomonas aeruginosa*, one of the major opportunistic pathogens producing infections in hospitalized and cystic fibrosis (CF) patients (3). CF is one of the most prevalent genetic inheritable diseases (4). People suffering CF have long-term *P. aeruginosa* chronic colonization of the bronchial tree (years), with recurrent infections that result in lung deterioration and finally in the death of the patient (5). Because these people are usually under antibiotic treatment, *P. aeruginosa* grows in their highly compartmentalized bronchial environment in the presence of gradients formed by widely variable concentrations of antibiotics. Learning the response of *P. aeruginosa* to subinhibitory concentrations of antibiotics is thus a relevant task to understanding the biological responses of this bacterium in patients under treatment (6). To that goal, we have developed a *P. aeruginosa* subgenomic DNA microarray containing 555 genes selected as relevant for the development of chronic colonization and infection, antibiotic resistance, transcriptional regulation, and stress response. The effect of three antimicrobial agents belonging to different structural families, namely tetracycline, tobramycin, and ciprofloxacin, on the transcription of virulence-related determinants of *P. aeruginosa* was analyzed by using this subgenomic array. To further understand the effect of antibiotics on *P. aeruginosa* pathophysiology, functional analyses on the production of a number of determinants relevant for the virulence of this pathogen also were performed.

Here we show that specific antibiotics at given concentrations may increase expression of bacterial virulence determinants. This result has clear implications for the treatment of infectious diseases. In addition, our results offer more information for understanding the ecological role of antibiotics in nature. In this regard, antibiotics are good examples of hormesis (7, 8). At high concentrations, they are bacterial killers, whereas at low concentrations, they produce specific changes that might eventually favor the behavior of susceptible bacteria in nature.

Results and Discussion

The concentrations of the antibiotics used for the transcriptomic studies were selected just below a decrease in the growth rate of *P. aeruginosa* was observed (arrows in Fig. 1*a*).

As shown in Tables 1 and 2, treatment with each antibiotic produced changes in the expression of a specific subset of genes, although there was a common type of response in some cases. Overall, tobramycin treatment induced the expression (fold change >2) of 9 of the 555 genes present in the microarray (1.6%) and repressed the expression of 31 (5.6%). Ciprofloxacin induced the expression of 8 (1.4%) genes and repressed the expression of 21 (3.8%). Finally, tetracycline induced the expression of 8 (1.4%) genes and repressed the expression of 18 (3.2%). It is known that quinolones trigger the SOS response and the expression of pyocin genes in *P. aeruginosa* (9). Thus, the

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Abbreviations: CF, cystic fibrosis; T3S, type III secretion; T3SS, T3S system.

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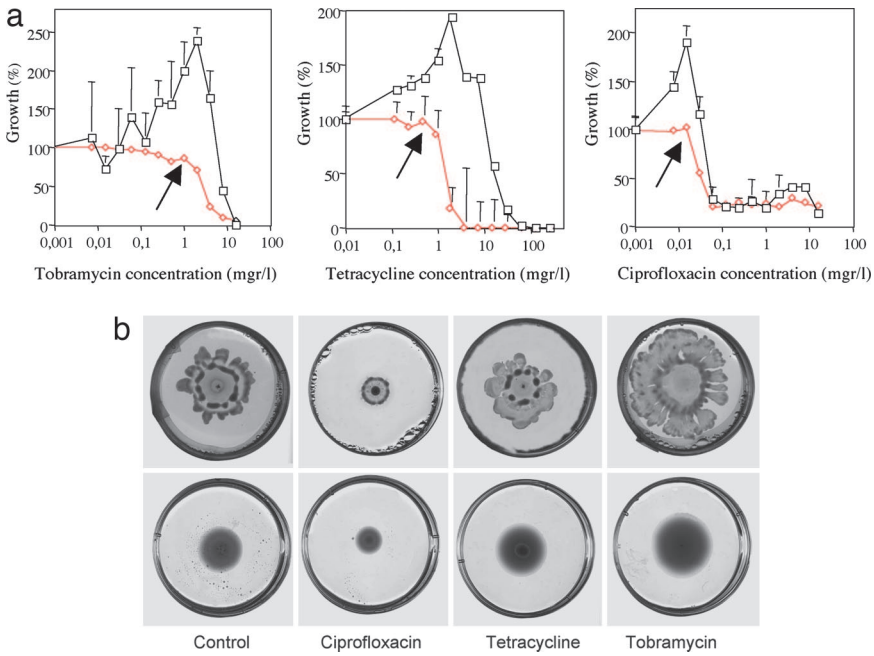

Fig. 1. Effect of antibiotics on biofilm formation and motility of *P. aeruginosa*. (*a*) The effects of different concentrations of antibiotics (tobramycin, *Left*; tetracycline, *Center*; ciprofloxacin; *Right*) on the formation of static biofilms was measured as described in ref. 33. Red, planktonic growth; black, growth-forming biofilm. The mean value and standard deviations of eight different replicates are shown. Standard deviation bar is not shown when its size was lower than the size of the symbol in the graph. The arrows indicate the concentrations of antibiotics used for the experiments with microarrays. (*b*) Effect of antibiotics in *P. aeruginosa* motility. Swarming (*Upper*) and swimming (*Lower*) motility was measured as described in ref. 34. Growth culture media were different as the one used in the biofilm assay and the experiments with microarrays, and thus antibiotic concentrations also were different. The concentrations used were 0.015 mg/liter ciprofloxacin, 0.5 mg/liter tetracycline, and 0.25 mg/liter tobramycin.

observed induction of those genes by ciprofloxacin (Table 1) confirms the reliability of our analysis.

Some of the genes with changes in their level of expression have a role in relevant traits for bacterial chronic colonization and virulence such as iron uptake, response to oxidative stress, motility, biofilm formation, and cytotoxicity. Thus, functional assays were preformed to address whether bacterial phenotypes changed accordingly. Noteworthy, the formation of static biofilm (Fig. 1*a*) increased when bacteria were incubated in the presence of all three antibiotics tested. Increase in biofilm formation attributable to tobramycin treatment has been reported recently and considered specific for aminoglycoside

Table 1. Genes up-regulated upon antibiotic exposure

		Fold change		
Gene number	Gene product	Tobramycin	Ciprofloxacin	Tetracycline
PA2019	Probable transcriptional regulator	**2.93**	*1.69*	**2.9**
PA0905	RsmA, regulator of secondary metabolites	**3.12**	1.26	−1.42
PA2399	Pyoverdine synthetase D	**2.37**	1	1.09
PA1704	Transcriptional regulator protein PcrR	**2.16**	*1.51*	−1.05
PA2397	Pyoverdine biosynthesis protein PvdE	**2.1**	1.13	−1.17
PA1528	Cell division protein ZipA	**2.06**	1.36	1.05
PA3007	Repressor protein LexA	**2.05**	**3.58**	−1.03
PA1151	Pyocin S2 immunity protein	**3.13**	**3.17**	1.48
PA3617	RecA protein	*1.65*	**2.63**	−1.01
PA1475	Heme exporter protein CcmA	*1.51*	**2.15**	−1.8
PA0610	Transcriptional regulator PrtN	*1.8*	**4.24**	−1.2
PA0985	Pyocin S5	*1.54*	**3.42**	1.9
PA1150	Pyocin S2	1.27	**3.59**	1.2
PA3866	Pyocin protein	−1.21	**16.28**	**2.03**
PA4228	Pyochelin biosynthesis protein PchD	−1.43	−1.54	**2.54**
PA4290	Probable chemotaxis transducer	1.16	−1.22	**2.53**
PA3326	Probable Clp-family ATP-dependent protease	1.06	1.06	**2.1**
PA1003	Transcriptional regulator	−1.09	−1.13	**2.1**
PA3327	Probable nonribosomal peptide synthetase	1.63	1.33	**2.04**
PA2532	Thiol peroxidase	1.02	−1.14	**2**

Bold indicates genes with fold change >2 or <−2 and with $P < 0.05$, and italic indicates genes with fold change >1.5 or <−1.5 and the $P < 0.05$.

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Table 2. Genes down-regulated upon antibiotic exposure

Gene number	Gene product	Fold change		
		Tobramycin	Ciprofloxacin	Tetracycline
PA0527	Transcriptional regulator Dnr	**−7.7**	**−3.18**	**−3**
PA4067	PAOuter membrane protein OprG precursor	**−5.74**	**−3.12**	**−2.11**
PA1561	Aerotaxis receptor Aer	**−4.2**	**−2.95**	**−2**
PA0403	Regulator PyrR	**−3.02**	**−2.3**	**−2.2**
PA5128	Secretion protein SecB	**−2.22**	**−2.41**	1
PA4407	PAcell division protein FtsZ	**−5.57**	**−3.25**	*−1.66*
PA0949	Trp repressor binding protein WrbA	**−3.02**	**−2.14**	1.5
PA1077	Flagellar basal-body rod protein FlgB	**−3**	**−2.45**	1.19
PA0928	Sensor/response regulator hybrid	**−3.9**	**−1.97**	−1.26
PA0958	Basic amino acid and imipenem porin OprD precursor	**−3.86**	**−2.08**	1.41
PA5563	Chromosome partitioning protein Soj	**−2.28**	**−2.21**	−1.01
PA4366	PAsuperoxide dismutase	**−2.07**	**−2.14**	1.17
PA0594	Peptidyl-prolyl cis-trans isomerase SurA	**−2.65**	**−2.03**	−1.32
PA0519	Nitrite reductase precursor	**−3.08**	*−1.97*	**−3.05**
PA0970	TolR protein	**−2.23**	*−1.69*	**−2.42**
PA0972	TolB protein	**−2.24**	*−1.94*	**−3.39**
PA1318	Probable chemotaxis transducer82–1431058 cytochrome *o* ubiquinol oxidase subunit I	**−2.19**	*−1.57*	**−5.09**
PA0516	Heme d1 biosynthesis protein NirF	**−4.82**	−1.22	**−5.47**
PA1094	Flagellar capping protein FliD	**−3.08**	*−1.82*	−1.53
PA1094	Flagellar capping protein FliD	−3.08	−1.82	−1.53
PA0024	Coproporphyrinogen III oxidase, aerobic	**−2.95**	−1.43	−1.68
PA0003	RecF protein	**−2.82**	−1.44	−1.59
PA4236	PAcatalase	**−2.77**	−1.46	−1.4
PA3999	PAD-ala-D-ala-carboxypeptidase	**−2.72**	*−1.53*	−1.09
PA1081	Flagellar basal-body rod protein FlgF	**−2.71**	−1.28	−1.24
PA0782	Proline dehydrogenase PutA	**−2.66**	−1.36	2.1
PA1092	Flagellin type B	**−3.04**	−1.45	−1.24
PA0004	Transcription antitermination protein NusG-6695 DNA gyrase subunit B	**−2.49**	−1.45	−1.16
PA4587	Cytochrome *c*551 peroxidase precursor	**−2.46**	−1.3	*−1.79*
PA4235	PAbacterioferritin	**−2.02**	*−1.74*	−1.33
PA4937	Exoribonuclease RNase R	**−2**	−1.16	−1.38
PA0969	TolQ protein	**−1.97**	−1.25	−1.56
PA1431	Regulatory protein RsaL	−1.29	**−2.9**	1.9
PA0763	Anti-sigma factor MucA	1.79	**−2.53**	−1.06
PA4386	PAGroES protein	−1.06	**−2.3**	−1.15
PA2853	Outer membrane lipoprotein OprI precursor	−1.58	**−2.26**	1.08
PA1912	Probable sigma-70 factor, ECF subfamily	*−1.57*	**−2.2**	1.43
PA5040	Type 4 fimbrial biogenesis outer membrane protein PilQ precursor	−1.41	**−2.15**	1.11
PA0831	Transcriptional regulator OruR	1.19	**−2.05**	−1.31
PA3397	Ferredoxin–$NADP^+$ reductase	−1.66	**−2**	−1.31
PA3529	Probable peroxidase	−1.48	**−2**	−1.1
PA3529	Probable peroxidase	−1.48	**−2**	−1.1
PA4403	PAsecretion protein SecA	−1.25	**−2**	−1.68
PA0764	Negative regulator for alginate biosynthesis MucB	1.2	1.23	**−3**
PA4761	DnaK protein	−1.02	−1.45	**−2.44**
PA5053	Heat shock protein HslV	1.11	−1.16	**−2.34**
PA1351	Probable sigma-70 factor, ECF subfamily	1.32	2.48	**−2.31**
PA0523	Cytochrome *c* subunit of nitric oxide reductase	2.61	1.14	**−2.18**
PA5242	Polyphosphate kinase	−1.75	−1.23	**−2.14**
PA0294	Transcriptional regulator AguR	−1.1	−1.58	**−2**
PA0857	Morphogene protein BolA	−1.54	−1.1	**−2**
PA0374	Cell division protein FtsE	1.1	1.79	**−2**
PA0518	Cytochrome *c*-551 precursor	*−1.93*	*−1.88*	**−2.89**

Bold indicates genes with fold change >2 or <−2 and with $P < 0.05$, and italic indicates genes with fold change >1.5 or <−1.5 and the $P < 0.05$.

antibiotics, because no effect was detected with members of other antibiotic classes, such as carbenicillin, chloramphenicol, or polymyxin (2). We confirmed that tobramycin induced the formation of biofilm and found that tetracycline and ciprofloxacin had the same effect at concentrations below their minimal inhibitory concentrations (MICs). Searching the literature suggests that a similar phenomenon may occur for other antibiotics. For instance, it has been shown that β-lactams induce the synthesis of colanic acid in *Escherichia coli* (10). Because colanic acid is involved in adhesion to surfaces

in this bacterial species (11), it can be predicted that β-lactams might induce biofilm formation in *E. coli*.

Most previously published works on the effect of antibiotics on bacterial physiology assay only one or just a few antibiotic concentrations, frequently above minimal inhibitory concentrations. In these cases, the observed changes deal mainly with the mechanisms involved in antibiotic-mediated growth inhibition or killing. However, we showed that lower (nonlethal) subminimal inhibitory concentrations of antibiotics might result in more subtle changes in bacterial physiology, eventually involving different ''signaling'' cell-to-cell effects with specific consequences in the collective behavior of the bacterial population. In fact, although the effect on biofilm formation seemed to be common for all three antibiotics tested, some other changes in bacterial physiology were antibiotic-specific. Swimming and swarming are two important systems of bacterial motility and probably related with the pathogenic process in CF patients. When the effect of antibiotics in these motility types was explored, clear differences were observed among the different antibacterials (Fig. 1*b*): We did not detect any effect on motility in the case of bacteria growing in the presence of tetracycline, whereas a reduction in both types of motility was observed in the case of ciprofloxacin. Noteworthy, the aminoglycoside tobramycin induced both swimming and swarming of *P. aeruginosa*. Again, this finding indicates that subinhibitory antibiotic concentrations do not necessarily produce a burden on bacterial physiology but in some occasions may enhance some potentially adaptive characteristics useful for colonization of specific environments. Indeed, in agreement with the idea that antibiotics might trigger some phenotypes that can be advantageous for the ecological behavior of bacterial populations, it has been described that subinhibitory antibiotic concentrations might increase mutation frequency in *P. aeruginosa* (12), favoring adaptive radiation (13) and allowing more efficient colonization of heterogeneous environments by this opportunistic pathogen.

P. aeruginosa strains can be either cytoinvasive or cytotoxic (14). Type III secretion (T3S) has a predominant role in the cytotoxic phenotype of this bacterial species. T3S is a system by which bacterial pathogens can deliver effectors directly into the cytoplasm of their eukaryotic host cell (15). The T3S system (T3SS) regulon is composed by different operons coding the proteins involved in the secretion and the effectors. Expression of the genes forming the regulon is triggered by ExsA (16), a transcriptional activator that autoregulates its own expression by a feedback mechanism. It is known that the expression of the T3SS is induced by the contact of the bacteria with its host cell. Calcium deprivation also induces T3S in *P. aeruginosa*. However, the physiological inductor of the expression of T3S remains unknown. Besides these specific inductors, it has been found recently that metabolic disbalance or overexpression of multidrug efflux pumps also might produce changes in T3S (17). Because antibiotics exposure was expected to produce a general burden to bacterial physiology that might affect T3S, we have measured the expression the gene *exsA*, coding ExsA, the transcriptional activator of the T3SS in *P. aeruginosa*, and the gene *exoS*, coding the T3SS-secreted exotoxin ExoS, in conditions that induce T3S. Surprisingly, incubation with tetracycline (1 mg/liter) increased the expression of T3S genes (Fig. 2*a*), whereas no changes were observed for the other antibiotics (data not shown). To confirm these results with an *in vivo* model, we measured the cytotoxicity produced by *P. aeruginosa* cells grown in the presence of antibiotics on a macrophage cell line. As shown in Fig. 2*b*, the presence of tetracycline increased cytotoxicity of *P. aeruginosa* by nearly 4-fold. We did not detect any effect when the added antibiotics were ciprofloxacin or tobramycin (data not shown). When tetracycline was added alone to the macrophage cell line, we did not observe any cytotoxic effect either, indicating that the effect is attributable to the bacteria growing in the presence of the antibiotic. Paradoxical increases in wound infection have been recorded during tetracycline therapy, which have been attributed to tetracycline inhibition of host wound-healing mechanisms (18, 19). Our observations might suggest that subinhibitory concentrations of tetracyclines also might increase bacterial pathogenesis. Quinolone antibiotics previously have been shown to increase toxicity in *Shigella*, but as a result of bacteriophage induction (20); our work is an example showing that subinhibitory concentrations of antibiotics may induce the expression of chromosomal genes encoding specific bacterial virulence factors.

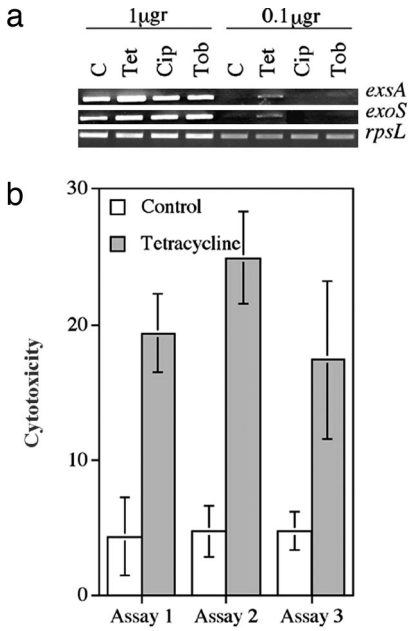

Fig. 2. Effect of tetracycline on the expression of T3SS and the cytotoxicity of *P. aeruginosa*. (*a*) The effect of tetracycline on the expression of the T3SS genes *exsA* and *exoS* was estimated by semiquantitative RT-PCR as described in ref. 17, by using *rpsL* as a control of RNA load. (*b*) The effect of tetracycline on the cytotoxicity of *P. aeruginosa* on a macrophage cell line was measured as described in ref. 17. Three experiments, each one consisting of eight replicates are shown. For each assay, median values and standard deviations are presented. White, cytotoxicity of *P. aeruginosa* without antibiotics; gray, cytotoxicity of *P. aeruginosa* in the presence of tetracycline.

An unexpected residual excess of teleological thinking has influenced the evolution of biological sciences during the last few decades. Although this approach can contribute to the establishment of potential scenarios of research, it frequently leads to wrong interpretations when applied to the experimental results. Antibiotics have been searched on purpose as inhibitors (either bactericidal or bacteriostatic) of bacterial growth, and we have tended to consider that microbes are using them to play the same function in nature. The automatic application of a classic Darwinian view supported the idea that such bacterial killer compounds could play an important role in the fight between microbial competitors for colonizing the same niche. It is obvious that many primary or secondary metabolites, not only antibiotics, might exert different effects at different concentrations. For instance, antibiotics that inhibit bacterial topoisomerases might, at low concentrations, produce changes in DNA supercoiling (21, 22). Because DNA supercoiling regulates expression of virulence determinants in some bacterial species (23), a general change in the virulent properties in the presence of subinhibitory concentrations of these antibiotics might be expected.

Our results support the notion that antibiotics provide a good example of hormesis (7, 8), as they have a very different effect on

susceptible bacteria depending on their actual concentration. The results shown in the present work and others (2, 24) indicate that subinhibitory concentrations of antibiotics can produce specific changes in the behavior of susceptible bacteria. In fact, it recently has been demonstrated that rifampicin induces specific modulation of transcription in *Salmonella typhimurium* in a promoter-dependent manner, without the need of global regulators or the bacterial global stress response (35). It has been stated that this could be just a defensive response to subinhibitory antibiotic concentrations (2, 25). Opposite to that view, we believe that these results, more than reflecting bacterial competition, reflect bacterial cooperation and offer more information on the actual ecological and evolutionary role of antibiotics in nature. Some arguments against the role of antibiotics as weapons were earlier raised, including the low concentrations of free antibiotics present in soils compared with laboratory conditions and the fact that the highest antibiotic production is usually at the stationary growth phase not at the exponential one, when competition for resources would expected to concentrate. Let us imagine that antibiotic-producing microorganisms in nature could serve as an organizing force in integrated microbial communities, helping, for instance, nonantibiotic members of the community to colonize surfaces (by biofilm formation) or to fight against predator Protistae (cytotoxicity). We thus could start to envisage a Copernican turn-about for the role of antibiotics in nature: from weapons involved in microbial struggle for life to collective regulators of the homeostasis of microbial communities. Weapons and signals always have been linked in nature [armament-ornament duality (26)]. Our results show that antibiotics constitute a paradigmatic example of this general evolution trend. This view is supported by other work in addition to ours (27) and also might result in a reconsideration of the unexpected, and therefore unexplored, effects on microbial biosphere resulting from the release of industrial antibiotics or biocides in nature (27, 28), including those used to feed animal for their ability to promote growth.

Materials and Methods

Development of a Subgenomic DNA Microarray for Analyzing the Expression on Genes Relevant for the Infection by *P. aeruginosa*. To analyze the expression of genes involved in virulence and antibiotic resistance of *P. aeruginosa*, we have developed a subgenomic DNA microarray useful for determining the expression levels of a subset of genes present in the chromosome of *P. aeruginosa* PAO1. For microarray development, we have used the genome annotation of this bacteria (www.pseudomonas.com). The four criteria for selection were as follows. (*i*) The genes included belonged to the categories of virulence, antibiotic resistance, regulation of transcription, response to stress, and communication. (*ii*) Usually, only one gene was included from each operon. In case of operons with several genes, two genes were included. (*iii*) Most genes belonged to the categories 1 (function experimentally demonstrated in *P. aeruginosa*) or 2 (function of highly similar gene experimentally demonstrated in another organism) as annotated on the *P. aeruginosa* genome. (*iv*) Some other genes previously shown to have a differential expression either in growth conditions or in mutant strains with a relevant role for *P. aeruginosa* pathophysiology were included. Following these criteria, 555 genes were selected, which represent a good coverage of the genes with a known role on infection by *P. aeruginosa*. The list of the PA numbers of the genes included in the array is shown in Table 3, which is published as supporting information on the PNAS web site. Once the genes were selected, the oligonucleotides specific for each of them were designed, and the microarray was printed as described below.

The microarray thus contains 555 oligonucleotides (50-mer) spotted in duplicate onto γ-aminosylane-treated 25 × 75-mm microscope slides, covalently linked to the slide with UV light and heat. The spots, 180 μm in diameter, are separated from each other by 265 μm. The oligonucleotides, designed by BioAlma (Madrid, Spain), are specific for each selected ORF of *P. aeruginosa*. Negative controls [spotting buffer: 50% (vol/vol) dimethyl sulfoxide] were spotted in duplicate at 10 positions evenly distributed throughout the printed area. The array was printed by Progenika Biopharma (Vizcaya, Spain).

Bacterial Strains and Culture Media. Cells were grown in LB broth without glucose (29) with aeration at 37°C. Growth was followed by measuring turbidity at 600 nm (A_{600}). For RNA purifications, 500-ml flasks containing 100 ml of culture medium were inoculated with *P. aeruginosa* PAO1 to reach a turbidity of 0.03, introduced in a rotary shaker operated at 220 rpm, and incubated again in the presence of the selected antibiotic for 4 h.

Three antibiotics belonging to different structural families, namely tetracycline, ciprofloxacin, and tobramycin, were chosen to analyze the effect of subinhibitory concentrations of antibiotics on the expression of *P. aeruginosa* genes relevant for infection. The concentrations of the antibiotics were selected just below a clear decrease in the growth of *P. aeruginosa* is observed (arrows in Fig. 1*a*).

RNA Purification. Total RNA was extracted by using the phenol/guanidine thiocyanate mix Tri Reagent LS (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Residual DNA was removed by treatment with DNA-Free (Ambion, Austin, TX). After discarding the presence of contaminating DNA by PCR, the samples were purified with RNeasy columns (Qiagen, Valencia, CA), which helped to eliminate the 5S rRNA. RNA integrity was checked by agarose gel electrophoresis.

Hybridization and Processing of Microarrays. Fluorescently labeled cDNA for microarray hybridizations was obtained by using the SuperScript Indirect cDNA Labeling System (Invitrogen, Carlsbad, CA), as recommended by the supplier. In brief, 20 μg of total RNA were transformed to cDNA with SuperScript III reverse transcriptase by using random hexamers as primers and including aminoallyl- and aminohexyl-modified nucleotides in the reaction mixture. After cDNA purification, the Cy3 or Cy5 fluorescent dyes (Amersham Biosciences, Piscataway, NJ) were coupled to the amino-modified first-strand cDNA. Labeling efficiency was assessed by using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Before the hybridization process, the microarray was blocked by immersion into a 50-ml Falcon tube containing 5× SSC, 0.1% (wt/vol) SDS, and 1% (wt/vol) BSA and preheated to 42°C. After 45 min at 42°C, the microarray slide was washed by a brief immersion into a Falcon tube containing H_2O at 22°C, followed by another immersion in isopropanol. The slide then was allowed to dry.

Equal amounts of Cy3- or Cy5-labeled cDNAs (≈300 pmol each), one of them corresponding to the control and the other one to the problem to be analyzed, were mixed and dried in a Speed-Vac. The sample was dissolved in 35 μl of a solution containing 50% (vol/vol) deionized formamide, 5× Denhardt's solution, 6× SSC, 0.5 (wt/vol) SDS, and 5% (wt/vol) dextransulphate, prefiltered, and preheated to 42°C. After 2 min at 90°C to denature the cDNA, the solution was applied to the microarray slide and covered with a 24 × 60-mm cover glass. The slide was introduced into a hybridization chamber and incubated at 42°C for 18 h, preserved from light. The microarray then was transferred to a Falcon tube containing 0.5× SSPE (1× SSPE contains 150 mM NaCl, 1 mM EDTA, and 11.5 mM NaH_2PO_4, pH 7.4) and 0.5% (wt/vol) SDS and preheated to 37°C. After eliminating the cover glass, the microarray was washed by gentle agitation for 5 min. The slide was transferred to a new tube with 0.5× SSPE/0.5% (wt/vol) SDS at room temperature and washed again with gentle shaking for 5 min. Similar washes were performed three times in 0.5× SSPE at room temperature and once in 0.1× SSPE. The microarray was allowed to dry and scanned in

a microarray scanner with green and red lasers operating at 543 and 633 nm, respectively, to excite Cy3 and Cy5. Images were taken at 10-μm resolution, and spot intensity was determined by using the software packages QuantArray 3.0 (PerkinElmer, Wellesley, MA) or Genepix Pro 5.0 (Axon, Sunnyvale, CA).

For each experiment, three independent biological samples were processed. For each of these samples, two independent RNA extractions were performed, and the RNAs were mixed in equal proportions to avoid as much as possible problems attributable to biological variability. By this procedure, the number of false positives (genes detected as differentially expressed that are do not really have a differential level of expression) are reduced to a minimum, although the number of false negatives (genes that are differentially expressed but are not detected with this technology) may increase (30). Three technical replicas were made for each experiment. Thus, the total number of microarrays analyzed for each experiment (including technical and biological replicates) was nine. The results for each replica (median intensity for each spot, minus background) were normalized by the Lowess procedure (31), after which all replicates were merged into a single data set by using the SOLAR software package for microarray data analysis (BioAlma). The absolute value of the *t* statistic (32) was calculated as a measurement of the probability that a gene is not differentially expressed (*P* value). The output file provides, among other data, the fold change and *P* values for each spot. An M/A plot of each data set was performed to have a global view of the differential expression ratios in each assay.

From the data obtained with the microarray, we have selected those genes with fold change >2 or <-2 and with $P < 0.05$. The genes whose expressions change accordingly to these criteria are indicated in boldface in Tables 1 and 2. For those genes, the table also includes the fold change produced by incubation with the other two antibiotics. In this case, if the fold change is >1.5 or <-1.5 and the *P* value <0.05, the results are indicated in italics.

Biofilm Formation and Motility. Biofilm formation was quantified as previously described (33). Briefly, overnight cultures of the bacterial strains were diluted by 1/100 in fresh LB broth, and 0.1 ml of those bacterial suspensions were poured in Falcon 3911 MicroTest III silicone flexible assay plates. The plates were incubated 24 h at 37°C without agitation. Bacteria were stained with crystal violet and rinsed thoroughly several times with water. The biofilm-forming bacteria were detached with ethanol containing Triton X-100 (0.25%), and the absorbance was determined at 560 nm. Eight different wells were tested in each experiment. Means and standard deviations were calculated with Microsoft Excel. Swarming and swimming motility was assayed as previously described (34).

RT-PCR. Bacterial cells growing in conditions that induce T3S (17) were collected, spun down at 4°C, and frozen in dry ice. Total RNA was extracted by using the phenol/guanidine thiocyanate mix Tri Reagent LS (Molecular Research Center). Residual DNA was removed by treatment with the DNA-Free kit (Ambion). RT-PCR assays were performed by using Ready-To-Go RT-PCR beads (Amersham Biosciences) as indicated by the manufacturer. The amplifications were performed by using primers specific for the selected genes (17) and two serial 10-fold dilutions of the RNA (1 and 0.1 μg). To ascertain that no residual DNA was present in the RNA preparations, PCRs were performed by using the same primers and overall conditions, except that no reverse transcriptase was added. Expression of the *rpsL* gene was measured as an internal control that ensured that equal amounts of RNA were used in all of the RT-PCRs performed. The reaction mixtures were incubated for 30 min at 42°C, followed by 10 min at 95°C and 30 cycles of 30 s at 95°C (for *rpsL* and *exsA*), 63°C (for *exoS*), and 1 min at 72°C, before the final 7-min elongation at 72°C.

Cytotoxicity Assays. The J774 macrophage cell line (17) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO, Carlsbad, CA). The macrophages were seeded in 96-well culture plates 20 h before infection at 10^4 cells per well. On the day of the experiment, the cells were washed carefully and infected with *P. aeruginosa* either in the presence or in the absence of antibiotic at a multiplicity of infection of 50. Eight wells were infected for each determination. After 360 min of infection, cytotoxicity was evaluated by using the Cytotoxicity Detection kit (LDH; Roche, Indianapolis, IN) in accordance with the manufacturer's instructions. One hundred percent cytotoxicity was estimated by lysing noninfected cells with 2% (vol/vol) Triton X-100. Means and standard deviations were calculated with Microsoft Excel.

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